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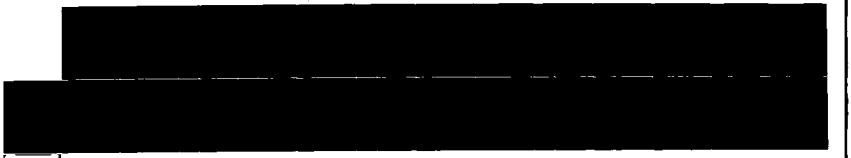

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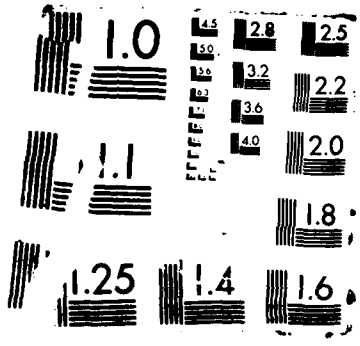
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RESEARCH AND DEVELOPMENT ON THE MODIFICATION OF STROMA-FREE HEMOGLOBIN FOR USE AS A BLOOD SUBSTITUTE

Annual and Final Report

A.G. Greenburg, M.D., Ph.D.

Paul W. Maffuid, M.S.

14 April 1984

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Chemical modification of stroma-free hemoglobin (SFH) using a series of ribose phosphates has been achieved. The P50 is 50-70% improved and the intravascular retention time increased by two to eight times (species dependent). The dial modified SFH has normal oxygen carrying capacity and maximally altered energetics. It is stable at 4°C liquid storage and maximum met-Hb is 4-5%. Details of the reactions indicate a significant yield (90% can be obtained by iteration of the reaction steps. Extensive purification is not needed.

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FOREWORD

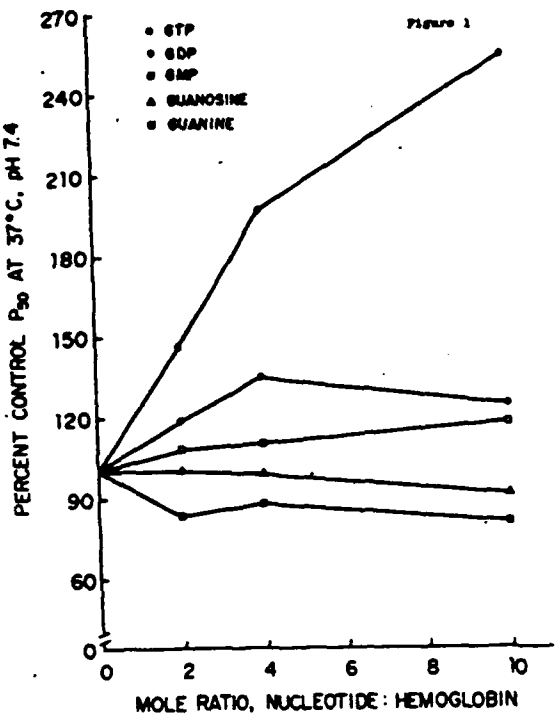
In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

THE PROBLEM AND BACKGROUND

The need for an oxygen carrying resuscitation fluid for use in trauma resulting in significant hypovolemia is not new (1). Since 1968, the potential for the use of stroma-free hemoglobin (SFH) has been improved. With the demonstration that true SFH was not toxic (the major toxicity coming from the stroma or red cell membrane fragments), evaluation using exchange models showed SFH capable of sustaining life in experimental animals. Two problems were apparent: a short intravascular retention time and an increased affinity of oxygen for SFH such that compensatory hemodynamic responses would be required. Thus began an era of chemical modification of SFH aimed at improving these characteristics (2,3). To improve one at the expense of the other did not seem reassurable. It was shown by 1977 that pyridoxalation of SFH improved both attributes in the desirable direction. Efforts were then directed at further intramolecular modification of SFH using analogs of DPG or other known allosteric effectors. The nucleotide polyphosphates demonstrate a significant allosteric effect on SFH. Oxidation of ribose phosphates, creating an "open ring dialdehyde", produces a molecule which can be bound to SFH improving both intravascular retention and oxy-Hb affinity. Improving the yield of this reaction and evaluation of other compounds has been the objective of this recent research effort.

Nucleotide Dialdehydes

Adenosine nucleotides exhibit an allosteric effect when added to human hemoglobin, the magnitude of effect is a function of the phosphate's size. ATP (5.5A), ADP (2.8A), and AMP (1.3A) have binding constants with hemoglobin that are lower than those of DPG (6.5A) (4) by one, two and three orders of magnitude respectively. The allosteric effectiveness of other nucleotides was demonstrated previously (please see figures 1,2). Recently, the periodate oxidation products of some ribose polyphosphates were considered as potential allosteric effectors and site specific crosslinking agents (5). However, the true nature of these products was never fully characterized and may have led to some of the variability in results. The oxidation of purine and pyrimidine ribonucleotides with periodate to give a reactive dialdehyde has been established as a convenient and efficient means of affinity labelling amino acids within nucleotide binding sites (6-13). The preparation and chemical properties of periodate oxidized adenosine triphosphate have been outlined (10) and indicate an instability towards beta--elimination in neutral or alkaline conditions. This work was used as a guideline for the preparation of 2'-o-[(R)-formyl (adenin-9-yl) methyl]-3'- triphosphate-3'-deoxy-(S)-glyceraldehyde (o-ATP). Other ribose derivatives were reacted under similar conditions. We will use o-RP when referring to the group of oxidized ribose phosphates. The uv properties are presented on table 1.



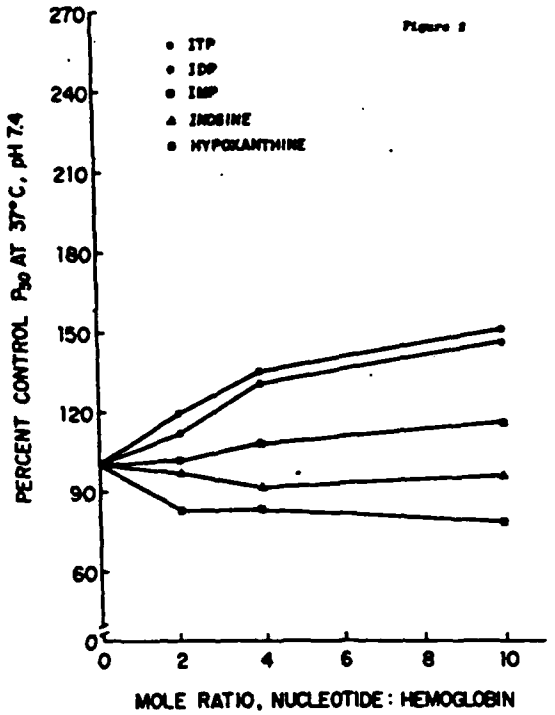
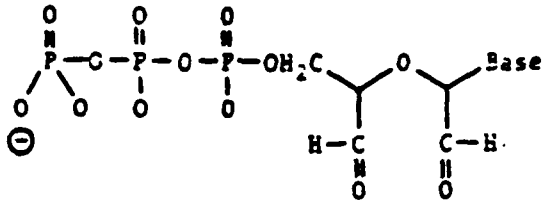


TABLE 1

Nucleotide Analog	Acidic Spectral Data pH	Max	Emax 10^{-3}	Min	$\frac{250}{260}$	$\frac{280}{260}$	Neutral Spectral Data pH	Max	Emax 10^{-3}	Min	$\frac{250}{260}$	$\frac{280}{260}$
Adenosine triphosphate	2	257	14.7	230	0.85	0.22	7	259	15.4	227	0.80	0.15
o-ATP	2	257	14.6	231	0.86	0.21	7	258	15.2	228	0.80	0.15
Guanosine triphosphate	1	256	12.4	228	0.96	0.67	7	253	13.7	223	1.17	0.66
o-GTP	1	256	12.3	227	0.95	0.66	7	253	13.5	220	1.20	0.68
Uridine triphosphate	2	262	10.0	230	0.75	0.38	7	262	10.0	230	0.75	0.38
o-UTP	2	262	10.2	231	0.72	0.36	7	262	9.8	229	0.77	0.40
Inosine triphosphate	2	250	12.2	226	-	-	7	248	8.2	222	1.68	0.25
o-ITP	2	249.5	12.1	225	-	-	7	248	8.1	223	1.64	0.21

Novel Dialdehydes

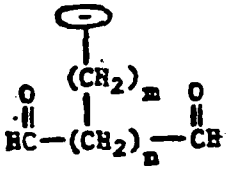
This portion of the project seeks to design and synthesize new and potentially valuable Hb crosslinking reagents based on the observed properties of SFH modified by periodate-oxidized ATP followed by in situ borohydride reduction as described elsewhere in this report. Oxidized ATP (o-ATP) is believed to crosslink the beta chains by Schiff base formations followed by:



o-ATP

Base = Adenine

irreversible hydride reduction. The triphosphate anionic portion of o-ATP generates the affinity for the cavity but the base would not appear to be necessary for binding. From the outset, therefore, the proposed crosslinking reagents were simple dialdehydes of varied length containing a suitably located negative charge:



From examination of models and consideration of synthetic ease the chain lengths of six (n=4) and eight (n=6) are our initial choices (c.f. o-ATP, n=3). The initial choice of negatively charged group was that of a monophosphate, recognizing that any compounds that showed promise, say by comparison with o-AMP, would ultimately require the synthetically more demanding triphosphate orienting group. Key in the choice of target compounds was the ready availability of starting materials and ultimate ease (low cost) of synthesis, recognizing that an expensive but inaccessible compound such as the Pocker dialdehyde (2-Nor-2-formylpyridoxal-5'phosphate), while of great theoretical interest, would be too expensive for practical use.

STATEMENT OF PROBLEM

The enzyme site specificity for adenosine triphosphate and its periodate oxidation product has wide documentation. Although the allosteric effect of ribose phosphates with hemoglobin is well known, only a brief description of the oxidations product's reaction with hemoglobin has been published. The use of hemoglobin

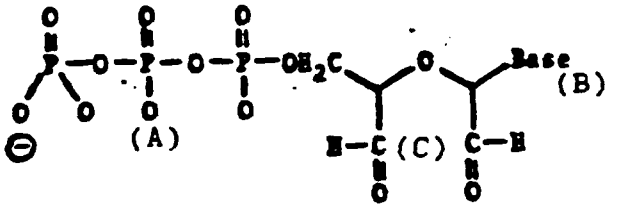
modified with these dialdehydes as a blood substitute has not been explored. The problem is one of:

I. Synthesizing a homogeneous dialdehyde phosphate.
II. Characterizing the dialdehyde to identify the nature of the relative species.
III. Reacting purified dialdehyde with hemoglobin under controlled conditions.
IV. Characterizing for amount of reaction and degree if any of crosslinking.
V. Purification of product or optimization of yields.
VI. Determination of physical and physiological properties and evaluating its usefulness as a blood substitute.

APPROACH

Characteristics of Periodate Oxidized Ribose Phosphates o-RP

The oxidation product of the ribose phosphates requires identification of three components:



o-ATP

Base = Adenine

[A] The degree of polyphosphate character - identifiable by electrophoresis.
[B] Comparison of uv spectra under standard conditions [220--300nm; 0.1N HCl, 0.1N NaOH].
[C] As the aldehydes are easily hydrated their spectroscopic properties may be complex, although a direct identification of a nucleotide dialdehyde by chromatography (11) has been reported. Easily characterized is the borohydride reduction product (14).

Reaction with Human Hemoglobin

The reaction of o-RP with hemoglobin involves the initial formation of a reversible (@ pH=7.4) imine, and reduction by sodium borohydride affording a covalent crosslink. Of interest is the degree of modification (A) and the degree of crosslinking (B).

(A) Cellulose acetate electrophoresis used for the deter-

mination of glycosylated hemoglobins and cation exchange HPLC conveniently measures changes in the isoelectric point of hemoglobin, indicative of modification.

(B) SDS-PAGE electrophoresis in conjunction with molecular weight standards was used to identify the degree of subunit crosslinking.

Approach/Vascular Retention Time

Methods of measuring vascular retention time are well documented (2,3,15). Half-life monitoring acts as an experimental in vivo indicator with respect to Hb-modification. Thus, the approach in vascular retention time analysis is to:

A. Interpret changes in retention time.
B. Identify physiological abnormalities inherent with exchange.
C. Adjust parameters to observe changes in vascular retention time.

EXPERIMENTAL

Adenosine triphosphate (Sigma, disodium salt, Grade I, 99%), adenosine monophosphate (Sigma, sodium salt, Type II, 99%) adenosine (Sigma), quanosine (Sigma), guanosine monophosphate (Sigma, sodium salt), quanosine triphosphate (Sigma, Type III), inosine (Sigma), inosine monophosphate (Sigma, Grade III), inosine triphosphate (Sigma, Grade I), uridine (Sigma), uridine monophosphate (Sigma, disodium), uridine triphosphate (Sigma, Type III), acrylamide (Sigma), bis-acrylamide (Sigma), tetramethylenediamine (Sigma), sodium periodate (Aldrich), and sodium borohydride (Aldrich) were used as commercially supplied. All other chemicals were reagent grade unless otherwise specified. Ozone was generated using an Ovec Model 03V5-0 ozonizer with P(02)=5psi, Fl=5 at 0.8 amperes. Sephadex gels were supplied through Sigma. BioRex-70 is a product of BioRad.

Uv and visible spectral data were obtained on a Perkin Elmer Lambda 3 spectrophotometer. Dissociation curves were plotted by a Hemoxanalyser with the optical system regulated to 20 +/- 0.1degC with a Neslab EX100 Exacal/EN150 Endocal System. P50 values were obtained at 20degC to minimize oxidation (16). Hemoglobin spectral characteristics were measured on the Instrumentation Laboratory IL282 automatic spectrophotometer and oxygen capacity was calculated from the relationship 02 capacity = 1.39 1-(% COHb + % MET Hb)/100, using values thus obtained. Direct measurement by catalytic conversion using a LexO2Con verified these results. Solution pH was measured on an Instrumentation Laboratory 813 pH/blood gas analyser. Osmotic pressures were read directly from a Wescor 5100-C vapor pressure osmometer. Inorganic ions (Na, K, Ca, Mg, CH3C00, Cl), the absence of aerobic or anaerobic bacteria

were provided by the Clinical Chemistry Division, VAMC, San Diego. Thrombin times were measured directly from thromboelastographic patterns obtained on a thromboelastograph D (Hellige). High performance liquid chromatography was performed on Waters Associates equipment consisting of two Model 6000A solvent delivery systems, Model 710A automatic injector, Model 440A dual wavelength detector filtered at 280nm or 405nm, Model 720 system controller and Model 730 data module. Cation exchange HPLC was afforded using a column manufactured by Synchrom Inc, Linden IN; a Synchropak CM 300 (Cat. # 103-25) (4.1mm x 250mm). Cellulose acetate electrophoresis was performed at pH 8.4 in tris-edta-borate buffer at 450v for 25 minutes as described by Helena. Electrophoretic mobility was referenced using a preparation of hemoglobins A,F,S, and C supplied by Isolab. Polyacrylamide gel electrophoresis was run through 10% continuous (pH=8.9) or 6-34% gradient gels in tris-glycine: Tris-HCl buffer (pH 8.9-8.1) all 1.5% in bis-acrylamide (17). Glutaraldehyde crosslinked hemoglobin (Sigma) was the molecular standard.

Preparation of o-RP

Periodate oxidation is essentially that described by Lowe and co-workers (10). In a typical reaction, a 20ml solution of ribose phosphate (0.4mM) and sodium periodate (0.38mM) at pH 6.8, 4degC, were stirred for one hour or until reaction was established complete by chromatography (please see table 2). Excess periodate was quenched by addition of ethylene glycol (20ul). Periodate salts were removed by gel filtration through a column of Sephadex G-10 equilibrated and eluted with water. Eluent was monitored at 254nm and 0.4ml fractions were collected. Fractions corresponding to the last 25% of the elution profile were discarded. The recovered yield was 75% based on absorbance (18) and verified homogeneous by chromatography. A lack of beta-elimination decomposition product was indicative of no absorbance maxima between 220 and 230nm. Solutions were stored at -78degC.

Preparation of 3-Hydroxymethyl Cyclohexene

A sample of 17.1g of 3-cyclohexenecarboxaldehyde was dissolved in 75ml absolute ethanol and cooled to 10degC. Solid sodium borohydride (4.0g) was added slowly with stirring. After stirring one additional hour at room temperature, 1.0g of additional sodium borohydride was added and the solution heated at reflex for 1.5 h. The solution was cooled and concentrated *in vacuo* to about half of the original volume. With cooling, concentrated hydrochloric acid was added dropwise to a pH of 1. Water (100ml) was added and the solution extracted three times with 200ml portions of ether. The combined ether extracts were washed once with concentrated potassium carbonate solution and dried over sodium sulfate. Filtration and concentration *in vacuo* gave 17.5g of a clear liquid which was fractionally distilled

TABLE 2

Compound	Rf Value a	b	c	d*
Adenosine	0.72	0.78	0.7	3
Adenosine monophosphate	0.42	0.17	0.2	12
Adenosine triphosphate	0.17	0.01	0.02	18
o-ATP	0.01	0.01	0.02	19
or-ATP	0.17	-	-	-
Guanosine	0.74	0.52	-	14
Guanosine monophosphate	0.39	0.12	-	14
Guanosine triphosphate	0.15	0.01	-	21
o-GTP	0.01	0.01	-	20
or-ATP	0.17	-	-	-
Inosine	0.81	0.62	-	6
Inosine monophosphate	0.44	0.17		18
Inosine triphosphate	0.14	0.01	-	
o-ITP	0.01	0.01	-	
or-ITP	0.14	-	-	-
Uridine	0.77	0.31	-	3
Uridine monophosphate	0.40	0.11	-	12
Uridine triphosphate	0.16	0.02	-	17
o-UTP	0.01	0.00	-	--
or-UTP	0.17	-	-	-
Phosphorylribose pyrophosphate	0.11+			
o-PRPP	0.00+			

Chromatography was performed on commercial sheets w/fluorescent indicator and irriadiated with uv light to afford detection. The systems are:

(a) Ascending chromatography on pei-cellulose F (Merck #5504) with 0.8 M ammonium bicarbonate.

(b) Ascending chromatography on cellulose (Eastman #6065) with the solvent system butanol-acetic acid-water (7:1:5),

(c) and with the solvent system iso-propanol-ammonium hydroxide-water (7:1:2).

(d) Electrophoresis was performed on Eastman cellulose sheets for 20 minutes at 800v using 0.02 M potassium dihydrogen phosphate.

* measured in cm from the anode.
+ detection by diazold spray (Sigma).

through a 2 inch Vigreaux column to give 15.1g (87%) of a clear liquid (b.p. 87-90 deg C (20mm)). The NMR spectrum shows broad absorption from = 5.64, 2 H).

Note: Use of methanol or isopropanol as solvent for this reaction yielded significantly less pure product.

Preparation of 3-Hydroxymethyl Cyclohexene Dihydrogen Phosphate (19)

4.0ml (0.044mol) of POCl3 were placed in a 100ml 3-neck round bottom flask outfitted with a stopper, an addition funnel, a magnetic stirbar, and an outlet leading to a water aspirator. The flask was placed in a room temperature water bath. 4.82g (0.043-mol) of 3-hydroxymethyl cyclohexene were added dropwise to the POCl3 with vigorous stirring. HCl(g) was removed by the aspirator. The mixture was stirred at room temperature for one hour. The water bath was heated to 50degC and the reaction mixture was stirred for four hours. The mixture was then added dropwise to 175ml ice water with vigorous stirring. The solution was allowed to stir for 4 1/2 hours. The solution was extracted with diethyl ether (1x100ml, 4.50ml). The ether layers were combined and washed with H2O (5x15ml) and dried over Na2SO4. The solution was filtered and the ether was evaporated on the rotary evaporator and the vacuum pump. Product was a thick yellow liquid. Yield = 6.42g. Further extractions of the aqueous layer with ether (2x100-ml) resulted in recovery of 0.75g. Total yield = 7.17g (87%).

Preparation of 3-Hydroxymethyl hexandial dihydrogen phosphate

A sample of 1.20g (0.0062mol) of cyclohexene 3-methyl phosphate was dissolved in 50ml methanol. A solution of 0.53g (0.0063-mol) of NaHCO in 8ml of water was added. A mixture of ozone and oxygen was bubbled through the reaction mixture for 25 minutes until a trap solution of potassium iodide turned orange and the reaction mixture became purple. The solution was flushed with nitrogen for 30 minutes at which time 1ml of (CH3)2S (0.015mol) was added. A flow of nitrogen was maintained for an additional 10 minutes. The solution was then stirred at 0degC for 20 hours. Repeated evacuation afforded a white hydroscopic solid which was used without further purification.

Preparation of 4-Cyclooctenylbromide

This compound was prepared according to the literature (20). The crude product, b.p. 95-99 (20nm) was shown by GLC to be a mixture of three products, the two minor of which had no olefinic absorption in the NMR. Passage of this crude mixture through a silica gel/silver nitrate column using hexane as the eluent gave early fractions rich in the minor impurities and later fractions that were shown by GLC to be greater than 95% pure 4-cycloocte-

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suscitated control (Group I) survival was 19% (3/16). Survival was higher for all resuscitated models that for controls ($p < 0.01$). Shed blood was not statistically better that RL or PRPP.

SUMMARY

Stroma free hemoglobin was modified with a series of phosphate dialdehydes for use as a blood substitute. Dialdehydes prepared from naturally occurring ribose phosphates by periodate oxidation were stable and easily reacted with hemoglobin. The dialdehydes were prepared in good yield and characterized by chromatography, nuclear magnetic resonance, and ultraviolet spectroscopy. The novel dialdehydes proved more troublesome and are subject to aldol condensation. Reaction with hemoglobin is dependent upon tetrameric conformation and the directability of both aldehyde moieties. o-PRPP gave fewer reaction products than the nucleotide phosphates. The C4 novel dialdehyde gave only 20% modification without crosslink and no substantial change in oxygen affinity. These modifications reduce the cooperative nature of the hemoglobin but increase the P50 and vascular retention time. Shock resuscitation with Hb-PRPP showed it similar to shed blood.

CONCLUSION

Chemically modified hemoglobins using periodate oxidized ribose phosphates and an organic dialdehyde phosophate were prepared. Modification of hemoglobin was a function of the phosphates directive effect upon the aldehyde. The effect of these modifiers on the allosteric site was verified by oxygen affinity in the presence and absence of DPG or IHP. Oxygen affinity and vascular retention were improved. Cooperativity and stability decreased. The lack of covalent restraint implied the possibility of an ionically stabilized derivative. However, the usefulness of these compounds seems somewhat limited by the detrimental pertubation introduced through dialdehyde modification.

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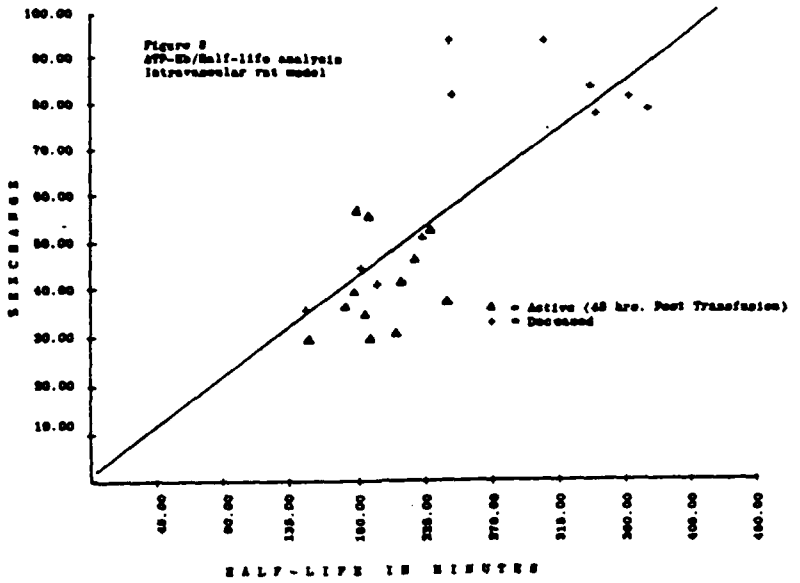


In other observations, the amount of urinary outputs for SFH and ATP rats were measured. In all cases the urine consisted primarily of hemoglobin. Furthermore, ATP-Hb rats excreted a larger volume of urine than the SFH rats. This is a distressing observation since, with a longer vascular retention time, one would expect ATP's urinary output to be less than SFH's.

Problems of vascular leakage, lethalities above 70% exchange, phsiological differences between SFH and ATP-Hb, and a diuretic effect of ATP-Hb are phenomena which merit additional study, and are in fact integral to the understanding of the hemoglobin picture. The results of shock resuscitation using our most successful modifier, PRPP, are seen below. The hemorrhagic shock model

GROUP	TREATMENT	# DEATHS @ 24 HR./ TOTAL RESUSCITATED	SURVIVAL %
I	CONTROL, NO RESUSCITATION	13/16	19%
II	SHED BLOOD (SB)	0/5	100%
III	LACTATED RINGERS, 2.5 x vol. SB	3/10	70%
IV	Hb-PRPP, 1 x vol. SB	3/10	70%

Hb-PRPP rats (Group IV) had a 48 hour survival (7/10) similar to RL-treated rats (Group III; 7/10) or whole blood treated rats (Group II; 5/5): 70%, 70%, and 100%, respectively. Nonre-

TABLE 4
ATP-Hb/Half-life analysis data

DATE	ATP-Hb Half-life	SFH (in minutes)	Length of experiment	%Exchange	Status
7-13	183.16		280 minutes	44.79	Deceased
7-14	146.08		180 minutes	35.71	Deceased
7-21	188.55			55.94	Alive
7-27	229.69			53.01	Alive
7-28	244.60		175 minutes	82.14	Deceased
	363.87		240 minutes	81.87	Deceased
8-2	148.01			29.82	Alive
8-3	209.98			41.86	Alive
	189.10			30.00	Alive
8-4	172.02			36.63	Alive
	206.48			31.11	Alive
8-9	193.85		265 minutes	41.25	Deceased
	178.07			39.74	Alive
8-10	240.55			37.50	Alive
	185.93			35.00	Alive
8-11	219.00			46.75	Alive
	224.34		255 minutes	51.16	Deceased
8-30		132.10		47.73	Alive
		104.30		47.73	Alive
8-31		117.80		54.16	Alive
		126.90		61.36	Alive
9-1		117.78		45.24	Alive
		121.31		55.06	Alive
9-4	242.30		190 minutes	93.75	Deceased
	306.15		200 minutes	93.75	Deceased
9-17		216.00	160 minutes	90.01	Deceased
		232.40	150 minutes	91.11	Deceased
		206.40		75.00	Alive
9-20	341.10		305 minutes	78.30	Deceased
	376.10		265 minutes	79.10	Deceased
	338.40		120 minutes	84.10	Deceased
10-6		361.50	210 minutes	90.50	Deceased
		207.50	210 minutes	90.50	Deceased
		216.90		87.50	Alive
		232.10	180 minutes	91.30	Deceased
11-9	179.40			57.80	Alive

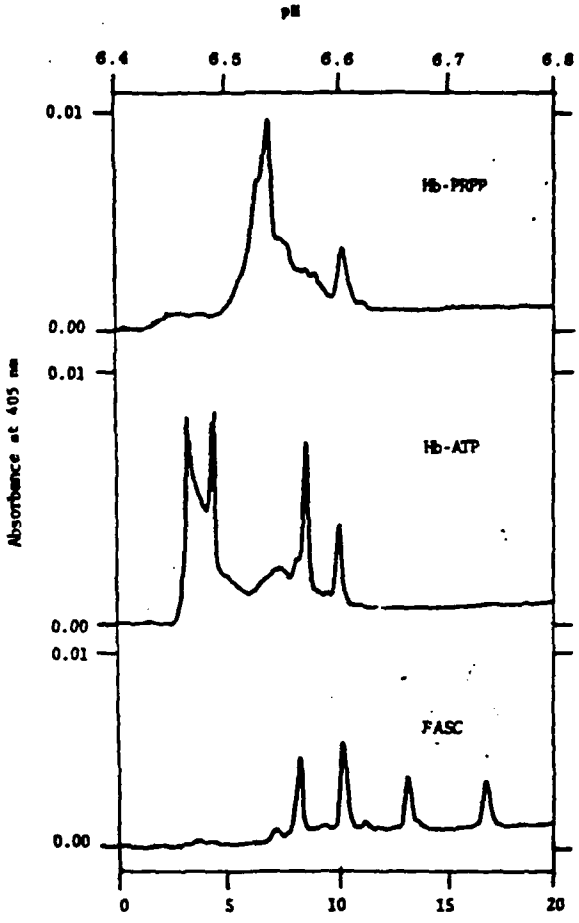
provides an excellent evaluation technique (see protocol). Half-life values for SFH and ATP-Hb at various % exchanges are shown on table 4. SFH has a mean half-life of 120.03 +/- 9.51 minutes at 50% exchange, and 238.97 +/- 55.03 minutes at 90% exchange. The mean half-life value at 90% differs with the findings reported by De Venuto (15). The mean half-lives for ATP-Hb are: 193.39 +/- 27.07 minutes at 50%, and 320.81 +/- 50.39 minutes at 90% exchange. PRPP-Hb was 222.79 +/- 48.96 at 50% exchange (n=7). Clearly, there is a difference in half-lives between the respective hemoglobins at specific percent exchanges. This phenomenon is unexplainable pending further investigation.

The differences seen in half-lives at various % exchanges led to the hypothesis concerning concentrational dependence of half-life. Figure 8 represents a basic family regression (BFR) of % exchange versus half-life for ATP-Hb. The line represents the average slopes of each documented rat, calculated by BFR. As shown, the graph confirms that half-life is proportional to intravascular Hb concentration. Also shown on figure 8 is the comparison of deceased versus alive rats. This difference was discussed earlier.

(A) is the Isolab standard and (B) is hemoglobin starting material, lot #16, supplied by the University of Minnesota. Modification (C) was far less extensive than seen in the previous cases. P50 (3.8 vs 3.5) and Hill coefficients (2.1 vs 2.3) essentially unchanged. This reaction was typical of a series (n=10) performed with this compound. The low stability of the dialdehyde prevented synthesis of the triphosphate. This further modification was to establish if the low degree of permitization is a function of phosphate or dialdehyde. However, based on the success of pyridoxal phosphate as a modifier one may attribute the low yield to the random nature of the crosslinking group. Successful modification apparently demands a directive group neighboring the covalent modifier. Of some concern were the results of the isopropanol stability test which reflected a substantial reduction by modification. Long-term storage reflected this result with an accumulation of precipitate.

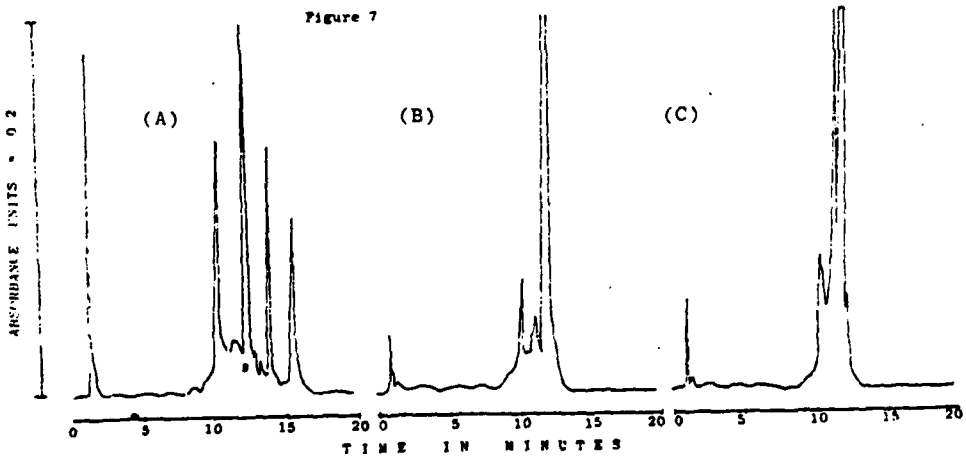
In vivo half-life studies of stroma-free hemoglobin (SFH) and chemically modified (o-ATP), (o-PRPP) were conducted. Prior to use, all solutions were monitored for viscosity, osmotic pressure, thrombodynamic potential, isopropanol stability, lipopolysaccharides, oxygen affinity, and were 7.0 to 7.7 g Hb/100ml of renal dialysis fluid. Earlier experiments in this product were inconclusive because of volume related complications. Progressive and irreversible shock due to apparent hypovolemia was of major concern in terms of circulating half-life or survivability (36). Therefore, each resuscitation fluid was evaluated at different % exchanges in opposition to exclusively 90% exchanges. In the data presented specific steps were taken to avoid hypovolemia and shock (37). The amount of shed blood per exchange was limited to 25% of the rodents' vascular volume (please see methodology). As a result of these measures, the survivability of transfused rats increased, while the effect on vascular retention time allowed specification of baseline values for unmodified material. Table 4 represents rats exchanged at 90% and 50% classes for both SFH and ATP-Hb. The fatalities represent rodents that did not survive past the 48th hour, post-transfusionally. SFH at the 90% exchange class had a 71.4% (n=7) fatality as opposed to no fatalities (n=6) at the 50% exchange class. ATP shows a similar pattern; 100% (n=7) fatality at 90% exchange, and 25% (n=6) at 50% exchange. There appears to be a fatality threshold associated with the percentage of exchange for both SFH and ATP (see also figure 8). An explanation would involve the possibility of hypovolemia resulting from decreasing vascular persistance of the material. Studies of decreasing Hb concentration with time (noting expired animals), have been documented. Further verification of the hypovolemia problem has been uncovered by preliminary autopsy studies by our lab on deceased rodents. Of all autopsies conducted, large amounts of Hb solution have been found in the peritoneal cavity indicating vascular leakage. To understand the hypovolemic condition associated with the hemoglobins, one must investigate its intravascular feasibility. Vascular half-life analysis

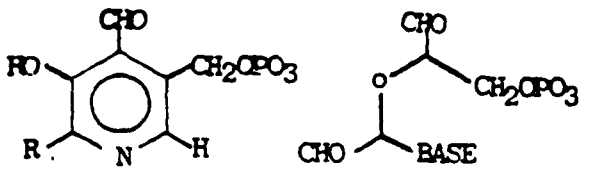
FIGURE 6



Conditions listed within the experimental.

Figure 7





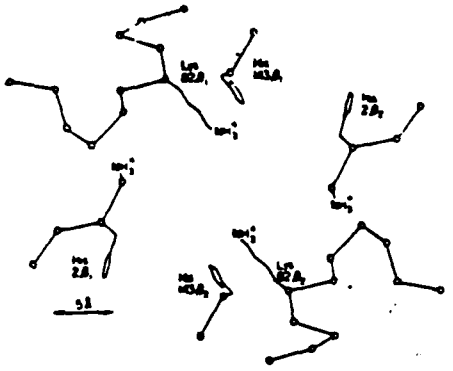
R=CH_3 Pyridoxal phosphate
R=CHO 2-Nor

group resulting from oxidation of the ribose ring is 7.6A. Wood and co-workers (33) reported from their study of the reaction of diaspirins with hemoglobin that the optimal span of flexible bridging groups is 6.5-8.0A. Therefore, from their results the reaction of o-ATP with hemoglobin should result in a modification which is 54% modified and bridges beta 1 lys 82 - beta 2 lys 82.

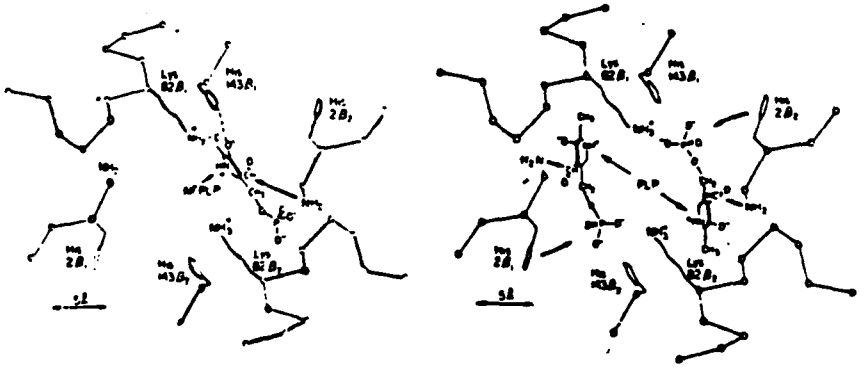
The chromatogram in figure 6 depicts the reaction profile of o-ATP (1.4eq) with deoxygenated hemoglobin. Phosphate analysis showed 2.7 moles phosphate/mole Hb tetramer. 10% SDS-PAGE indicated the modifiction contained 14% crosslinked material. The oxygen binding studies showed this solution to contain a modification which reduces the oxygen affinity and cooperativity. This loss in cooperativity appears to be function of subunit pertibation also reported with the bridging agent, bis-(N-maleimidomethyl) ether (34). Reaction of the remaining nucleotide dialdehydes provided multiple products similar to ATP, without substantial crosslinking. From molecular models we postulated that the sterically large base was directing the 2'-aldehyde to a region remote from amine functionality. Although not covalently crosslinked, this modification did result in a substantial increase in the plasma retention time.

To investigate on our speculation of base steric interference, we investigated the reaction of periodate oxidized phosphoryl ribose pyrophosphate (o-PRPP) with hemoglobin. Identical conditions were employed to afford production of dialdehyde and hemoglobin modification. The chromatogram in figure 6 illustrates the greatly improved reaction specificity. However, the results of molecular filtration indicated that intersubunit crosslinking was not achieved. The P50 value was slightly improved over the nucleotides, however, the Hill coefficient again reflected a loss in cooperativity. Vascular retention was improved over both unmodified hemoglobin and Hb-ATP. The novel dialdehydes were to investigate the reactivity of a sterically unopposed moiety. Reaction of the dialdehyde would be random, similar to glutaraldehyde, but limited to the allosteric region by the phosphate group's affinity. Octandial 4'-phosphate would serve to investigate a 10.2A crosslinking agent; perhaps additional flexibility would decrease the loss in cooperativity. The reductive amination of deoxy hemoglobin with BMP was by standard conditions. 10eq of dialdehyde were used to compensate for the lower association constant of monophosphates (35). The chromatogram in figure 7 is an HPLC analysis of the product mixture.

From the binding properties of adenosine triphosphate (29) with hemoglobin, it was established that ATP occupies the same binding site as DPG, and binds in the ratio: 1 mole ATP/mole DPG. That a single product would result from the reaction of o-ATP with hemoglobin is dependent upon the spatial orientation of the aldehyde moieties with neighboring amines. This orientation therefore is limited by the aldehyde bond length and conformational flexibility. As born out by the efforts of the Benesches and coworkers, phosphate containing molecules lacking the charge distribution of DPG bind in a manner dictated by pKa and steric orientation of the binding moiety. Shown below is the beta 1--beta 2 interface specific for binding of DPG, C02, Cl-, and phosphates. The relative binding distances are included. Pyridoxal phosphate



showed specificity for valine 1 or lysine 82 of the Beta subunits (30,31). 2-nor-2-formylpyridoxal 5'-phosphate binds by bridging valine 1 and lysine 82 of adjacent globins (32). The pyridoxal



compounds and the general candidate compound are diagrammed below. Interestingly, the dialdehyde carbon skeleton of the 2-Nor compound is identical in number to the candidate dialdehydes. This compound served as a constrained model for comparison to the flexible ribose dialdehydes. The span of the dialdehyde bridging

TABLE 3

	P_{50}			Hill Coefficient							
Hemoglobin	-	2mM DPG	2mM IHP	-	2mM DPG	2mM IHP	N	XL	S	t 1/2	O_2
Hb-SF	3.4	11.4	38.0	2.7	2.8	2.7	2	-	8	106.12 ± 12.57	1.37
Hb-ATP	7.4	7.8	7.6	1.9	1.8	1.9	3+	12	20	193.39 ± 27.07	1.45
Hb-GTP	7.1	7.4	7.2	1.8	1.9	1.9	3+	10	22	-	1.39
Hb-ITP	7.5	7.7	7.9	1.9	1.9	1.9	3+	13	19	-	1.40
Hb-UTP	6.9	7.1	7.1	1.8	1.7	1.8	3+	11	24	-	1.40
Hb-PRPP	9.2	9.4	9.3	1.8	1.7	1.8	2+	14	22	222.79 ± 48.96	1.46

N = The # of chromatographic species
XL = The % of hemoglobin determined to be crosslinked
S = The % of hemoglobin deemed unstable by isopropanol buffer (28)
t 1/2 = The vascular half-life
O_2 = The oxygen combining capacity

affinity for oxygen of modified hemoglobins resembled (B) in figure 5.

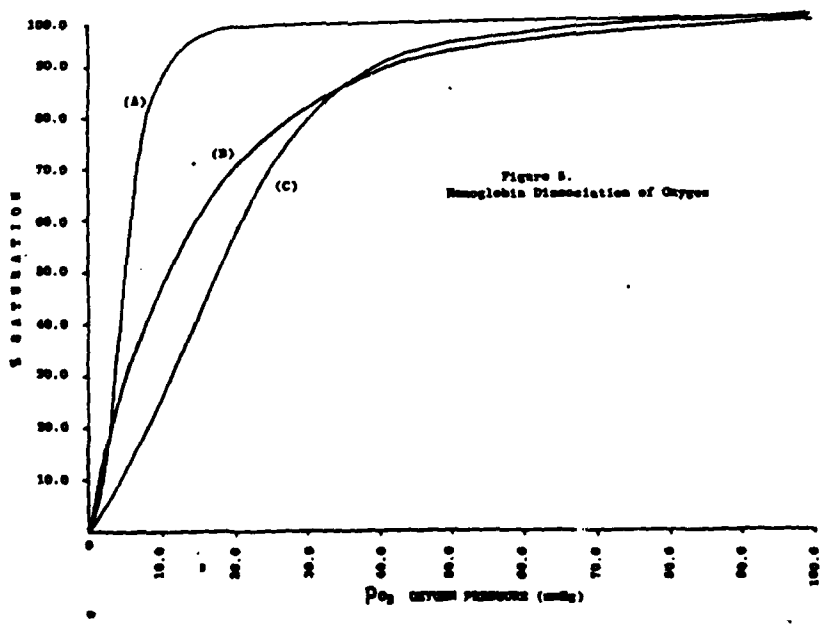
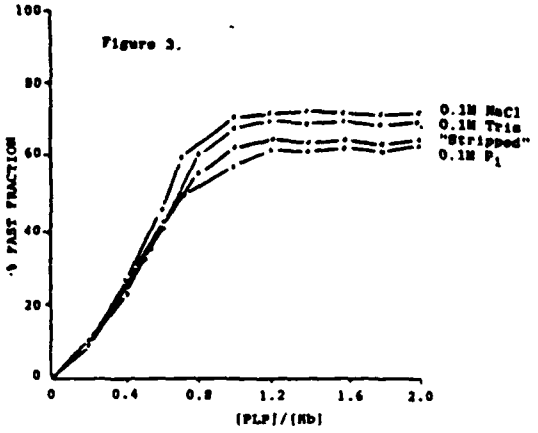
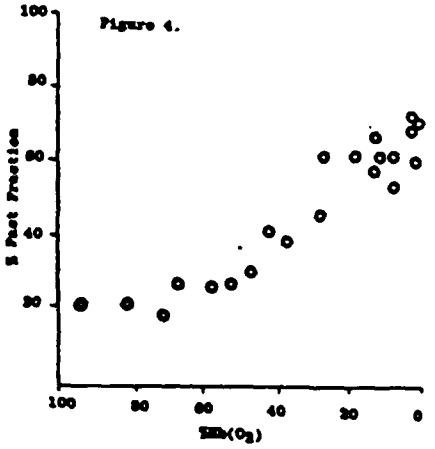


Figure 5.
Hemoglobin Dissociation of Oxygen

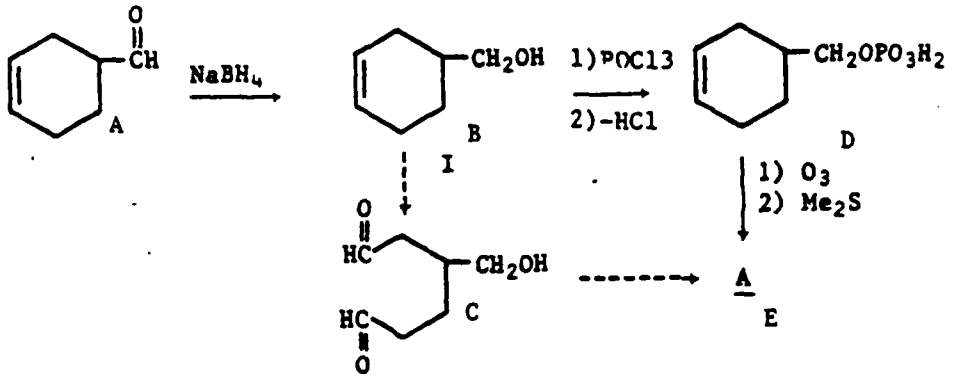
within the same range; 60-70% determined using cellulose acetate electrophoresis. This was surprising considering the reported transimination (25) reaction, and may be a function of polyphosphate character. Reaction of hemoglobin of various oxygen (please see figure 4) tensions with 1-2 equivalents of o-ATP



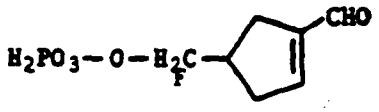


indicated the importance of efficient oxygen removal. Even fair yields (40%) are not obtained until the solution is less than 20% oxygenated. (Typical is the presence of 4-7% met.) Respectable yields (55-65%) were not obtained until more complete, less than 4%, deoxygenation was accomplished. At low oxygen concentrations, 2%, the presence of 4-7% met dictates an R state conformation of about 10%. This appears to be unsatisfactory for completion of this type of modification. The degree of modification has a maximum at 1.2 equivalents of dialdehyde, and 74% modification. Yields are increased through repeated reaction of these reduced/oxygenated/dialysed/deoxygenated solutions with dialdehyde. Three successive modifications increase yields upwards to 90%. SDS-page electrophoresis of this material reflects two bands, one at MW-17,500, the 2nd at MW-35,000 indicating modification is intramolecular and presumably within the Beta 1 and Beta 2 regions. This technique does not, however, detect intrasubunit modification.

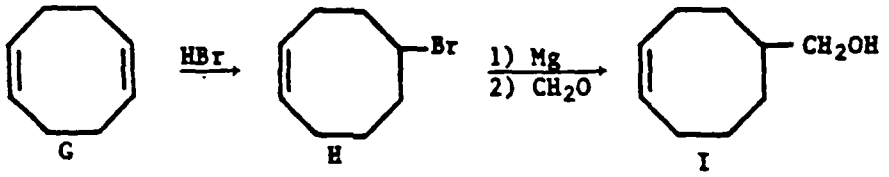
The dissociation curves of SFH (A), SFH-ATP (B), and SFH = IHP (C) (2E-4M) at 20DegC are shown in figure 5.Solutions were 0.1M tris, 0.1M NaCl, 60E-6M Hb, at pH=7.4. The SFH-ATP was a homogeneous fraction from cation exchange chromatography (26) (Trivelli, Ranney, Lai) and was reduced (27) prior to use. The P50 values are 5.0, 11.0, and 17.4 respectively. Table 3 provides a complete comparison of oxygen binding for the respective modified hemoglobins. Variation in P50 for Hb-SF and Hb-ATP is attributed to buffer and sample preparation. Table 3 reflects conditions to which all comparisons should be made. In all cases, the



ot compound C forced abandonment of Route I. Completion of the synthesis was through 1) phosphorylation of B to provide D in excellent yield followed by ozomolysis with reductive workup. The dialdehyde is unstable in solution undergoing an aldol reaction to compound F seen below assigned by NMR and uv.



The synthesis of octandial 4'-phosphate paralelled the above work. Due to the instable nature of the above dialdehyde, it was deemed appropriate to study the problem prior to committing the alcohol (I) to oxidation.



This work is in progress.

Prior to use with candidate dialdehydes, each lot of hemoglobin was reacted with pyridoxal phosphate by literature procedure (24). In all cases, reaction proceeded to a solution containing multiple products with P50 = 7.2-7.5 (7.8 w/2mM DPG).

The reaction of o-ATP with hemoglobin was used as model for the other nucleotides. Reaction with hemoglobin was examined for the influence of solvent (buffer), stoichiometry, and tetramer conformation (please see figure 3). There were four buffering systems: NaCl (0.1M), tris (0.1M pH=7.4), phosphate (0.1M, pH--7.4), and "stripped", a solution dialysed against distilled H20. The reaction yields were variable within each system but fell

were prepared by Sephadex G-10 chromatography in Developer A, pH 7.0. Chromatograms were at ambient temperature, 23-25degC. Columns were equilibrated in 95% A for 15 minutes prior to application of sample. A linear gradient from 5 to 95% in B in 15 minutes with isocratic development at 95% B for 5 minutes was used to elute hemoglobin samples. The flow rate was 2ml/min at 2300 psi. Absorbance was measured at 405nm. Columns were reequilibrated to 5% B after each run by a one minute gradient and isocratic conditions for 15 minutes.

Vascular Exchange

250-350 gram Sprauge-Dawley rats were anesthetized with sodium nembutal solution at a dosage of 50mg/kg. After rendering the animal unconscious, an incision was made on the left ventral side, proximal to the left leg for femoral artery isolation and cutdown. Upon isolation, the distal end was ligated and the artery perforated to accept a cannula. The cannula used was Silastic medical-grade tubing (0.020 in I.D. x 9.037 O.D.) manufactured by the Dow Corning Company. Cannulation was preceded by injection of 100 units of heparin (100 units/2 hours).

Transfusion of the studied materials began by withdrawing 5ml of blood (rate=5ml/minute), and immediately replacing it with 5ml of the resuscitation fluid. This procedure continues until the desired % exchange is achieved. This time is T=0.

Measurement of the Hb vascular retention time began by withdrawing 0.3ml of vascular fluid and spinning down the platelets in solution. The serum is then taken and analyzed for hemoglobin concentration using the cyanamethemoglobin technique (22,23). This procedure is repeated four times, at t=0, t=120, t=210 and t=240 minutes. Rodents were sterilly sutured with a running closure in two layers using 3/0 silk. Half-lives were calculated from the slope of -lnAbs vs time.

RESULTS AND DISCUSSION

The preparation and purification of all oxidized ribose phosphates followed from the procedure developed for adenosine triphosphate (10,11). Periodate oxidation provided a facile method for preparation of dialdehydes in the range of pH 6.5-6.8. Reaction is easily followed by TLC, and Sephadex G-10 chromatography affords a product free of salts (uv vs anhydrous weight). Chromatography of the aldehyde reduction product (14), the 2,3-dialcohol (or-RP), provided a simple test to establish the integrity of the dialdehyde moiety. Beta-elimination would have resulted in a product with Rf=0.9 in solvent system A. In all cases, the product resulting from periodate oxidation and borohydride reduction was homogeneous as one spot on TLC.

Synthesis of the novel dialdehydes proved to be a formidable task. Two routes for the synthesis of 3-hydroxymethyl dihydrogen phosphate (HMP) are seen below. The unsuccessful phosphorylation

nylbromide. The yield of product was not maximized in this preliminary experiment.

Preparation of 4-Cyclooctene-1-methanol

4-Cyclooctenylbromide (1.89g, 0.01 mole) in 10ml of anhydrous ether was added dropwise to a stirred suspension of magnesium (0.48g, 0.02 mole) in 25ml dry ether. The reaction was slow to begin and initiation was facilitated by the addition of one drop of 1,2-dibromoethane. The reaction became exothermic for about ten minutes and then warmed at reflex for 30 minutes. After cooling, the ether solution was added slowly, with stirring and cooling to a solution of paraformaldehyde (4-fold excess) in ether. The resulting milky white solution was poured onto ice, acidified to pH 1 with 10% HCl, extracted three times with ether and worked up in the usual manner to yield 1.95g of a light yellow liquid, molecularly distilled (100degC,2mm) to give 1.27g of clear liquid, n25D 1.4924 (lit. 1.4930). The infrared and NMR spectra were consistent with the assigned structure.

Preparation of Hemoglobin

Solutions of hemoglobin were provided by the Department of Surgery, University of Minnesota, Minneapolis, Minnesota. Prior to use, they were freed from phosphate and other ions by passage through a column of Sephadex G-25 equilibrated and eluted with buffer of the appropriate reaction medium at 4degC. Solutions were used immediately after preparation. Hemoglobin concentrations were determined by 1.1E4/heme at 540nm (MW=64,458) (21) and by an automatic spectrophotometer (IL282). pH measurements were determined on an IL813.

Covalent Modification of Hemoglobin

Hemoglobin (1mM) at pH 7.3 was deoxygenated by consecutive cycles of evacuation of 10E-3mm Hg followed by a purge with purified nitrogen. Deoxygenation was verified with an automatic spectrophotometer (IL282). The required amount of o-RP (10mM) in buffer was degassed by evacuation and added under a positive pressure of nitrogen. The solution was stirred 30 minutes, reduced by an addition of sodium borohydride (12 eq in 1mM NaOH), and after continued stirring for 30 minutes was oxygenated. Unreacted o-RP and borate salts were removed by dialysis against renal fluid or by passage through a column of Sephadex G-25 equilibrated with renal fluid.

Cation Exchange HPLC

The developers were: A) 0.04M bis-tris, 0.004M KCN, pH 6.1; B) A + 0.2M NaCl, pH 6.8. Solutions were filtered (0.45u) prior to use and used within three days of preparation. Hemoglobins


DAT
FILM